MECHANISTIC STUDIES OF COPPER(II) AMINOGLYCOSIDE MEDIATED DNA DAMAGE AND MAGNESIUM CATALYZED NUCLEASE ACTIVITY OF HAMMERHEAD RIBOZYME

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the Graduate School of The Ohio State University

By

Anjali A. Patwardhan, M.S.

*****

The Ohio State University

2003

Dissertation Committee

Professor James A. Cowan (Adviser)
Professor Sheldon Shore
Professor Claudia Turro

Approved by

Adviser

Department of Chemistry
ABSTRACT

The antibacterial activity of aminoglycosides stems from their high affinity binding to the 16S rRNA in bacteria resulting in inhibition of protein synthesis. Used to treat acute bacterial infections these antibiotics have limited applications due to their high dosage requirements and the emergence of resistant strains. We have synthesized and characterized the Cu(II) derivatives of six aminoglycosides. Kanamycin A, tobramycin and neamine exhibit preferential and tight binding to Cu(II) whereas neomycin B, kanamycin B and paromomycin don’t bind as tightly. Frozen solution EPR and UV-visible spectroscopy suggest a change in geometry around the Cu(II) but the stabilities of the complexes in water differ. These copper derivatives efficiently cleave plasmid DNA at micromolar concentrations (hydrolytic) and at nanomolar concentrations in the presence of co-reactants such as hydrogen peroxide or ascorbic acid (oxidative or radical mediated). Hydrolysis is multi turnover and exhibits Michaelis-Menten kinetics with enzyme-like behavior whereas oxidative cleavage is highly specific with C-4’ H abstraction resulting in characteristic base propenal and nucleotide base products.
Freeze-trapped EPR samples show the presence of radical species, and room temperature trapping by 2, 2’- dimethyl pyroline oxide confirms the presence of hydroxyl radicals in reactions containing hydrogen peroxide. Spin integration of EPR spectra of frozen samples obtained by mixing the complexes treated co-reactant shows the presence of a diamagnetic species in equilibrium with a paramagnetic species. The hydroxyl radical generated is not diffusible in the presence of substrate, which suggests that it may be copper based and is generated in close proximity to the substrate. This controlled formation of hydroxyl radicals may explain the high specificity exhibited by these complexes.

Hammerhead ribozymes are selectively hydrolyzed in the presence of divalent ions with Mg$^{2+}$ being the metal ion of choice \textit{in vivo}. The mechanism of hammerhead cleavage has been a subject of extensive research for the past two decades and a model that conforms to all results is yet to emerge. Our studies with complex ions such as cobalt hexaammine and \textit{fac}-triamminetriaquochromium(III) establish that Mg$^{2+}$ must interact with the hammerhead in the catalytic site via outer sphere coordination of its bound water molecules. There are essentially two sets of sites, one structural and one catalytic. The structural site needs to be occupied for activity and corresponds to the high affinity site. Complex ions in the catalytic site and divalent ions in the structural site result in a slow but active hammerhead ribozyme suggesting that the complex ions are not inhibitory, contrary to what was suggested previously.
Dedicated to my parents whose encouragement and support made this possible.
ACKNOWLEDGMENTS

I wish to thank my adviser, Jimmy Cowan for his patience, intellectual support and trust during the course of my PhD. I am grateful for the independence I was given in my work and am happy and proud to be a member of his lab.

I would like to say special thanks to Sheref for the many scientific discussions we have had over the years over countless cups of coffee. They have enriched my experience as a graduate student and I appreciate his friendship. I am indebted to Shumin and Sreedhara for their help in the early stages of my Ph.D. My present and past lab members have made my stay pleasant and I thank them for their support, help and understanding. I would like to thank Craig Hemann and Prof. Russ Hille for help with EPR, Karl Vermillion for NMR and Kari Green Church and Susan Hatcher for Mass Spectrometry.

I would like to thank my family for their constant encouragement before and during my stay at Ohio State. I appreciate Milind’s support and patience for being by my side during this time.
Last but not the least I would like to thank the Department of Chemistry for providing me with financial support as a Teaching Assistant for the time I have been here and lending me the opportunity to be a part of this department.
VITA

January 9, 1974…………………….Born – Mumbai, India
1994…………………………………B. S. Chemistry, Bombay University
1996…………………………………M.S. Inorganic Chemistry, IIT, Bombay, India.
1996-1997………………………..Research Associate, Center for
                            Environmental Science and Engineering,
                            IIT Bombay, India
1997- 2003………………………….Graduate Teaching Assistant, The
                            Ohio State University

PUBLICATIONS

1. Sreedhara, A., A. Patwardhan, and J.A. Cowan, Novel reagents for targeted
cleavage of RNA sequences: towards a new family of inorganic pharmaceuticals.

2. Patwardhan, A. and J.A. Cowan, Highly specific oxidative damage of double-
strand DNA by copper aminoglycosides. Chemical Communications (Cambridge,

FIELDS OF STUDY

Major Field of Study: Chemistry

Division of Inorganic Chemistry
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Dedication</td>
<td>iv</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>v</td>
</tr>
<tr>
<td>Vita</td>
<td>vii</td>
</tr>
<tr>
<td>List of Tables</td>
<td>xi</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xii</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>xv</td>
</tr>
</tbody>
</table>

## Chapter

1. Syntheses and characterization of copper aminoglycosides
   1.1 Overview
   1.2 Strategy
   1.3 Introduction
2.5 Quantitation of cleavage for duplexes and plasmid DNA..........................59
2.6 Conclusions.................................................................61

3  Role of metal ions in the catalysis of the hammerhead ribozyme...............63
   3.1 Introduction.............................................................63
   3.2 Experimental............................................................70
      3.2.1  Materials and methods.........................................70
      3.2.2  Kinetics in the presence of divalent ions....................70
      3.2.3  Kinetics in the presence of complex trivalent ions and Mg$^{2+}$ or Mn$^{2+}$......71
   3.3 Results and discussion................................................72
   3.4 Conclusions.............................................................81

References.................................................................82
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Elemental analysis of complexes 1-6</td>
</tr>
<tr>
<td>1.2</td>
<td>UV-visible spectral parameters of complexes 1-6</td>
</tr>
<tr>
<td>1.3</td>
<td>EPR parameters of complexes 1-6 measured at 20K</td>
</tr>
<tr>
<td>1.4</td>
<td>$^{13}$C-$T_1$ relaxation measurements of kanamycin A and tobramycin in the presence of 60 $\mu$M Cu (II)</td>
</tr>
<tr>
<td>1.5</td>
<td>$^{13}$C-$T_1$ relaxation measurements of neamine in the presence of 60 $\mu$M Cu (II)</td>
</tr>
<tr>
<td>1.6</td>
<td>$^{13}$C-$T_1$ relaxation measurements of neomycin B in the presence of 150 $\mu$M Cu (II)</td>
</tr>
<tr>
<td>1.7</td>
<td>$^{13}$C-$T_1$ relaxation measurements of paromomycin in the presence of 150 $\mu$M Cu (II)</td>
</tr>
<tr>
<td>1.8</td>
<td>$^{13}$C-$T_1$ relaxation measurements of kanamycin B in the presence of 150 $\mu$M Cu (II)</td>
</tr>
<tr>
<td>1.9</td>
<td>$K_d$'s for aminoglycoside binding to Cu$^{2+}$ as monitored by charge transfer transitions</td>
</tr>
<tr>
<td>3.1</td>
<td>Apparent binding affinities for the divalent ions in the presence of 0.5 mM complex ions 1 and 2</td>
</tr>
<tr>
<td>3.2</td>
<td>Rate constants as measured for complex ions 1 and 2 in the absence of divalent ions</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Structures of aminoglycosides belonging to the kanamycin and neomycin class of antibiotics</td>
</tr>
<tr>
<td>1.2</td>
<td>Aminoglycosides bind (A) R23; (B) TAR; (C) RRE, RNA motifs with high affinity</td>
</tr>
<tr>
<td>1.3</td>
<td>Charge transfer transitions (A) and d-d transitions (B) for the six copper aminoglycosides. 1; CukanA, 2; Cuneam, 3; CutoB, 4; CukanB, 5; Cuparo, 6; CuneoB</td>
</tr>
<tr>
<td>1.4</td>
<td>(A) Hyperfine features associated with copper (I=3/2) binding to kanamycin A (1) (B) X-band EPR of complexes 2-6</td>
</tr>
<tr>
<td>1.5</td>
<td>$^{13}$C-T$_1$ relaxation spectra of Cu$^{2+}$ (60 µm) and kanamycin A (100 mM) in 100 mM HEPES, pH 7.9. The delay time’s range from 10 s to 1 ms (A-H)</td>
</tr>
<tr>
<td>1.6</td>
<td>(A) Copper binding to kanamycin A, kanamycin B and tobramycin monitored using a charge transfer transition for each of the copper-aminoglycosides at 243, 250 and 245 nm respectively. (B) Copper binding to neomycin B, paromomycin and neamine monitored using a charge transfer transition for each of the copper-aminoglycosides at 250, 255 and 230 nm respectively</td>
</tr>
<tr>
<td>2.1</td>
<td>X-band EPR spectrum of CukanA (blue) at 20 K in the presence of p(dA)-p(dT) (red), and p(A)-p(U) (green)</td>
</tr>
<tr>
<td>2.2</td>
<td>(A) EPR spectrum of CukanA (green) and CukanA + H$_2$O$_2$ (1:2) (red) 16 msec. (B) CukanA + Ascorbate (1:2) (red), 16 msec</td>
</tr>
</tbody>
</table>
2.3 Spin trapped hydroxyl radicals are represented by the signature spectrum obtained (green) by reaction with DMPO………………..44

2.4 HPLC traces of reaction mixture with 100 mg plasmid, 0.5 mM CukanA, 5 mM H₂O₂ incubated at 37°C, (A) without heating at 95°C; (B) after heating at 95°C for 10 min………………..48

2.5 Thiobarbituric acid assay for 100 µg of plasmid DNA (pUC19) + 0.5 mM CukanA + 5 mM ascorbate (A); + 5 mM H₂O₂ (B)………………51

2.6 C-1’ H abstraction leads to the formation of 2’-deoxyribonolactone if attacked by hydroxyl radical or 1’,2’-dehydrornucleotide intermediate…………………………………………………………………..52

2.7 Thiobarbituric acid assay for reactions with p(dA)-p(dT). 50 µg of duplex was treated with either 0.5 mM CukanA or Cuneam and 5 mM peroxide or ascorbate for 2 h at 37°C followed by the addition of 400 µL of 0.6% TBA and boiling at 95°C for 20 min……………………54

2.8 Orientation of the H atoms in the major and minor groove of DNA. The H atoms represented by the wedges point into the minor groove. C-1’, C-4’, C-5’ are secondary carbon atoms and are more susceptible to abstraction by a nearby radical………………………………………57

2.9 Netropsin inhibition of the homopolymeric duplexes in the presence of CukanA and hydrogen peroxide………………………………..58

2.10 (A) Calibration plots for the four DNA bases A, G, T and C obtained from plotting peak areas versus concentration (mM). (B) Beer’s law graph for TBA assay obtained by using malondialdehyde as standard……………………………………………………60

3.1 Hammerhead ribozyme (34 mer) and the 5’ fluorescein labeled substrate (13 mer)……………………………………………………..64

3.2 Structures of trivalent ions hexaamminecobalt (III), [Co(NH₃)₆]³⁺, 1; fac- triamminetriaquochromium (III), fac-[Cr(NH₃)₃(H₂O)₃]³⁺…………………………………………………………………………………72

3.3 A plot of k⁺obs versus divalent metal ion concentration for the hammerhead cleavage reaction………………………………………………………74
3.4 A plot of $k_{obs}$ versus divalent metal ion concentration, fitting
carried out using non interacting site equation..................................75

3.5 (A) Titration of Mg$^{2+}$ into, 5 µM substrate + 20 µM ribozyme + 0.5 mM 1 or 2. (B) Titration of Mn$^{2+}$ into, 5 µM substrate + 20 µM ribozyme + 0.5 mM 1 or 2 ...............................................................78
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DMPO</td>
<td>Dimethyl pyroline oxide</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-[2-Hydroxyethyl] piperazine-N’-2[ethanesulfonic acid]</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance Spectroscopy</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RRE</td>
<td>Rev Response Element</td>
</tr>
<tr>
<td>TAR</td>
<td>Trans Activating RNA</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>Tris (hydroxymethyl) Aminomethane Hydrochloride</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
</tbody>
</table>
1.1 Overview

Substitutions of the 2-deoxystreptamine ring by sugar rings with vicinal amine and hydroxyl groups results in a class of compounds that are called aminoglycosides and are used clinically to treat Gram negative bacterial infections. Compounds obtained from substitutions at the 4 and 6 positions (of the deoxystreptamine ring) with amino sugar rings result in the kanamycin class of compounds whereas substitutions at the 4 and 5
positions result in the neomycin class. **(Figure 1.1)** The sugar rings have amino groups that exhibit different pK_a’s ranging from 5.6 to 9.5. The aminoglycosides thus carry a positive charge under physiological conditions (pH 7.4). The pK_a of amine groups on ring A is the most basic (~9.6) whereas the ones on ring B (~5.6 and ~8.0) and ring C (~7.5) are closer to physiological pH.

The presence of these positively charged amino groups makes these aminoglycosides electrostatically favorable for binding to polyanionic species like nucleic acids. The –NH_2 and –OH groups give it tremendous potential for hydrogen bonding. Isothermal calorimetric studies of the binding of neomycin B with a 23 nucleotide long RNA stem loop show predominant hydrogen bonding contributions in addition to electrostatics.¹ The two rings A and B are implicated in binding to the nucleic acid and the others remain pendant.

The mode of antibacterial activity stems from their high affinity and selective interaction to the 16s RNA in bacteria. This interaction leads to inhibition of the protein synthetic apparatus in the bacteria leading to their destruction.
Figure 1.1: Structures of aminoglycosides belonging to the kanamycin and neomycin class of antibiotics. Rings A and B are considered to bind RNA whereas Ring B in kanamycin class and rings C and D in neomycin class remain pendant.
Bacterial resistance arises from expression of aminoglycoside modifying enzymes e.g. acetyltransferases, adenylyltransferases and phosphotransferases that transfer functional groups like acetyl, adenyly or phosphate to key functionalities on the sugar rings of antibiotics such as kanamycins, neomycins, neamine, paromamine, butirosin, ribostamycin, lividomycin, gentamicin B, geneticin, isepamicin and amikacin. The phosphotransferases transfer a terminal phosphate group from ATP onto the 3’ hydroxyl of the aminoglycoside. This selective labeling interferes with the electrostatic and hydrogen bonding interactions, dropping their affinity for nucleic acids leading to bacterial resistance to the antibiotic. A variety of derivatives of aminoglycosides that are resistant to modifications by these enzymes (part of the modification machinery of resistant strains) are being synthesized. Neamine being one of them is a derivative of neomycin B where the two rings A and B are retained and the other portion is cleaved by acid methanolysis. Paromamine is similarly a derivative of paromomycin. Amikacin is a derivative of kanamycin A where the B1-NH₂ is modified by acylation with 4-amino-2-hydroxybutyric acid. Kanamycin B is modified at the 6’-NH₂ group on ring A, generating an amino acid derivative at this position. In addition to antibiotic resistance, high dosages of aminoglycosides lead to hearing loss in susceptible individuals. Amikacin which was synthesized to combat gentamicin resistance, leads to auditory and nephro-toxicity at high dosages. These result in a narrow therapeutic window of blood plasma concentrations thereby reducing the use of these antibiotics in life threatening
situations. The high dosages and the occurrence of resistant strains make application of these antibiotics very limited.

Aminoglycosides are known to bind to various RNA molecules, including ribosomal RNA,\textsuperscript{5,6} group I introns,\textsuperscript{7,8} and the hammerhead ribozyme.\textsuperscript{9} They also inhibit binding of the HIV-1 Rev protein to its viral RNA\textsuperscript{10} and the Tat derived peptide to TAR RNA.\textsuperscript{11} These motifs have no sequence homology, but exhibit common structural characteristics namely a bulge, stem loop etc. (Figure 1.2)

Alternatively different classes have differing affinities for the same motif e.g. neomycin B binds the Rev Response Element with micromolar affinity whereas kanamycin A does so with a hundred micromolar affinity.

A structure function relationship for these aminoglycosides has not yet been established that relates all these various aspects of binding to the target. The essential requirement for such high affinity is the electrostatic and hydrogen bonding potential that these classes of antibiotics carry.

Aminoglycosides are therefore very attractive targets for development as therapeutics. Their potential to bind various RNA motifs with high binding affinity is especially important since they can be specifically targeted to them without damaging the rest of the cell. The problems associated with these drugs include resistance and cytotoxicity, which are not unlike other pharmaceuticals. Aminoglycosides are the only known sugar derivatives that bind to RNA and do so with a very high affinity. These factors make aminoglycoside antibiotics interesting from a scientific perspective. They also have
potential therapeutic uses and slight changes or derivatization of these antibiotics may result in the next generation of highly potent and less cytotoxic therapeutic agents.

**Figure 1.2:** Aminoglycosides bind (A) R23; (B) TAR; (C) RRE, RNA motifs with high affinity. All three RNA molecules have similar structural motifs and no sequence homology.
1.2 Strategy:

We have endeavored to generate a new class of antibiotics, namely copper aminoglycosides and to develop them as therapeutics using the aminoglycoside to selectively target various RNA motifs and utilize the copper center to do chemistry on the target. These should not only bind the target, but also destroy it and leave little or no possibility for developing resistant strains of bacteria. The interactions of Cu(II) with aminoglycosides have previously been studied in situ and there is precedent to support the idea that chelate formation stabilizes the binding of the metal ion to the antibiotic. Our preliminary studies with plasmid DNA hydrolysis showed these derivatives to be the most active. Selective acetylation or acylation of the aminoglycosides has been achieved by protection of certain amino groups by Cu$^{2+}$. The vicinal –OH and –NH$_2$ groups can complex with a metal ion and form a chelate, leaving the other amino groups open to derivatization. The copper complexes, in spite of being studied in situ, had never been isolated and so we chose to isolate the corresponding copper complexes of six aminoglycosides namely kanamycin A (kanA), kanamycin B (kanB), neomycin B (neoB), neamine (neam), tobramycin (tobr) and paromomycin (paro).
1.3 Introduction

The interaction of metal ions with aminoglycosides was previously studied in the context of their use for preferential N-protection to generate acyl derivatives of aminoglycosides. These studies utilized the fact that a metal ion anchoring onto an amine group can be further stabilized by chelation via a vicinal hydroxyl group (protonated or deprotonated). These chelates are stable and their solution structure characterization has been done extensively utilizing uv-vis, EPR and CD spectroscopy. The underlying theme in all of these studies has been the formation of chelate complexes of aminoglycosides such as kanamycin B and tobramycin with Cu(II), and the involvement of rings A and C in coordination. The effectiveness of binding is dependant on the presence of a vicinal group to generate a chelate. Monodentate complexes undergo hydrolysis above pH 7.0 and are not as stable. In all of our studies copper binding to aminoglycosides was found to result in the formation of 1:1 metal ligand complex. There are multiple vicinal –OH and –NH₂ (differing in pKₐ’s) groups on the various rings, which may lead to different modes of binding. The aminoglycosides within a certain class themselves differ from each other in subtle ways. These changes can have large effects as far as Cu²⁺ binding is concerned. A thorough syntheses and characterization of the copper aminoglycoside complexes has been documented in the following sections.
1.4 Methods and materials.

1.4.1 Materials.

Kanamycin A (kanA), kanamycin B (kanB), neomycin B (neoB), paromomycin (paro) and tobramycin (tobr) were purchased from Sigma Chemical Co., and used as received. Neamine (neam) was synthesized following literature procedures. The purity of neamine was confirmed by $^1$H, $^{13}$C-NMR and elemental analysis.$^{15}$

1.4.2 Elemental Analysis:

Elemental analysis was done at Oneida Research Incorporated and the molecular formula calculated from the analysis. (Table 1.1)

1.4.3 Electron Paramagnetic Resonance spectroscopy:

EPR spectra were recorded on a Bruker ESP 300 electron paramagnetic Resonance spectrometer equipped with an ER 035 NMR gauss meter and a Hewlett-Packard 5352B microwave frequency counter. Temperature control was achieved using an Oxford Instruments ESR 900 continuous-flow liquid helium cryostat and an ITC 4 temperature controller. All spectra were obtained at
20.0 K using a microwave power of 10 mW, a frequency of 9.4830 GHz, a modulation amplitude of 1.008 G and a modulation frequency of 100 kHz. The concentration of all samples was 10 mM.

1.4.4 UV-visible Spectroscopy:

UV-visible spectra were obtained on a HP 8453 at 25ºC, over the wavelength range of 200 nm to 1100 nm. All samples were prepared in water with concentrations around 20 mM to detect d-d transitions. For charge transfer transitions the samples were diluted to around 0.5 mM for Cu-kan A, Cu-neam and Cu-tobr and to around 1 mM for Cu-kan B, Cu-neo B and Cu-paro.

1.5 Synthesis of copper aminoglycosides.

Copper derivatives of neomycin B, kanamycin A, kanamycin B, paromomycin, tobramycin and neamine were synthesized, isolated in solid state and characterized by EPR, UV-visible spectroscopy and elemental analysis. Neamine was prepared by the acid catalyzed hydrolysis of neomycin B following published protocols. 15,16
1.5.1 Synthesis of Cu-kanamycin A, 1:
To kanamycin A sulfate (0.1455 g, 0.25 mmol), in 5 mL water, was added CuSO₄ (0.0624 g, 0.25 mmol). The reaction was stirred at room temperature for 24 h resulting in a blue colored solution. To this, ethanol (5 mL) was added to precipitate a blue solid, which was filtered, washed twice in ethanol by stirring for 6 h each time, dissolved in water, and ethanol precipitated, to yield a pure compound 1. TLC was carried out with a mixed solvent system of propanol: acetone: 20% aqueous NH₄OAc: NH₄OH (1:1:5:0.025) to give an \( R_f = 0.33 \) for 1 versus \( R_f = 0.60 \) for kanamycin A sulfate.

Syntheses of copper complexes of kanamycin B, neomycin B, neamine, paromomycin and tobramycin were carried out under similar reaction conditions and purification procedures. All complexes were obtained in pure form and characterized for their C, H, and N content by elemental analyses.

1.6 Characterization of the isolated Copper-aminoglycosides

1.6.1 Elemental Analyses
Elemental analyses were carried out and the appropriate molecular formula was calculated for the copper complexes. Table 1.1 lists all of the complexes with their molecular formulae.
<table>
<thead>
<tr>
<th>Complex</th>
<th>Experimental</th>
<th>Calculated</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>Rf</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%C</td>
<td>%H</td>
<td>%N</td>
<td>%C</td>
<td>%H</td>
<td>%N</td>
<td></td>
</tr>
<tr>
<td>CukanA (1)</td>
<td>24.56</td>
<td>5.80</td>
<td>6.18</td>
<td>24.89</td>
<td>5.99</td>
<td>6.45</td>
<td>0.33</td>
</tr>
<tr>
<td>[(C₁₅H₃₈N₄O₁₁Cu)(SO₄)₂].7H₂O</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CukanB (2)</td>
<td>23.66</td>
<td>5.30</td>
<td>7.48</td>
<td>23.71</td>
<td>5.42</td>
<td>7.72</td>
<td>0.30</td>
</tr>
<tr>
<td>[(C₁₅H₄₁N₅O₁₀Cu)(SO₄)₃].4H₂O</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CuneoB (3)</td>
<td>25.30</td>
<td>6.05</td>
<td>7.43</td>
<td>24.78</td>
<td>5.92</td>
<td>7.54</td>
<td>0.25</td>
</tr>
<tr>
<td>[(C₂₃H₅₀N₆O₁₃Cu)(SO₄)₃].8H₂O</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cutobr (4)</td>
<td>24.03</td>
<td>4.97</td>
<td>7.63</td>
<td>24.13</td>
<td>5.51</td>
<td>7.85</td>
<td>0.30</td>
</tr>
<tr>
<td>[(C₁₅H₄₉N₅O₁₃Cu)(SO₄)₃].4H₂O</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cuparo (5)</td>
<td>26.54</td>
<td>5.51</td>
<td>6.42</td>
<td>26.46</td>
<td>5.50</td>
<td>6.74</td>
<td>0.28</td>
</tr>
<tr>
<td>[(C₂₃H₄₉N₅O₁₄Cu)(SO₄)₃].4H₂O</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cuneam (6)</td>
<td>23.92</td>
<td>5.66</td>
<td>8.87</td>
<td>23.93</td>
<td>5.98</td>
<td>9.30</td>
<td>0.40</td>
</tr>
<tr>
<td>[(C₁₂H₂₂N₄O₄Cu)4H₂O.4HCl]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 1.1:** Elemental analyses of the complexes 1-6
1.6.2 UV-Visible Spectroscopy.

The uv-visible spectra of the complexes in water show d-d transitions (~ 700 nm) in addition to charge transfer transitions (below 300 nm) (Figure 1.3, Table 1.2). The spectra can be attributed to a tetragonal copper system. The d-d transitions identified below ~700 nm (655 nm, in CukanA) imply a chelate with NH₂ and O⁻ ligands.¹³ The other d-d transitions fall either around 700 nm (for Cuneam and Cutobr) or at a slightly longer wavelength of ~ 750 nm (for CukanB, CuneoB and Cuparo) indicating either a weak chelate in the former or the absence of one in the latter. Distinct ligand to metal charge transfer transitions are seen in the UV region of the spectrum.

Thus electronic spectroscopy confirms the ligation of copper to the various aminoglycosides. Complexes 1-3 exhibit similar characteristics and suggest similar binding modes, and 4-6 can be grouped together due to their similar spectral features. As will be apparent in the following sections this is a repetitive occurrence where 1-3 and 4-6 show similar behavior for most of the characterization techniques.
<table>
<thead>
<tr>
<th>Complex</th>
<th>$\lambda_{\text{max}}$ nm ($\varepsilon, \text{M}^{-1}, \text{cm}^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu-kan A</td>
<td>655 (31.5)$^a$, 243 (1408), 213 (2075)</td>
</tr>
<tr>
<td>Cu-kan B</td>
<td>753 (17), sh 250 (663)</td>
</tr>
<tr>
<td>Cu-neam</td>
<td>704 (62), sh $\sim$ 230 (2973)</td>
</tr>
<tr>
<td>Cu-neo B</td>
<td>724 (17), sh $\sim$ 250 (448)</td>
</tr>
<tr>
<td>Cu-paro</td>
<td>757 (15), sh 255 (405)</td>
</tr>
<tr>
<td>Cu-tobr</td>
<td>703 (27), sh 245 (1453)</td>
</tr>
</tbody>
</table>

sh: shoulder; a: d-d transitions indicative of a chelate (-NH$_2$, -OH)

**Table 1.2: UV-visible spectral parameters**
Figure 1.3: Charge transfer transitions (A) and d-d transitions (B) for the six copper aminoglycosides. 1: CukanA, 2: Cneam, 3: Cutob, 4: CukanB, 5: Cuparo, 6: CuneoB in water. Concentrations of 20 mM were used to detect d-d transitions. CukanA, Cuneam and Cutobr were diluted to 0.5 mM whereas CukanB, CuneoB and Cuparo were at 1 mM each to detect charge transfer transitions.

1.6.3 EPR spectroscopy:

Copper(II) with a d⁹ system and nuclear spin of I=3/2 gives rise to a signature four line EPR signal. The coupling within the parallel components is characteristic and dependant
on the coordination and geometry of the ligands around the copper. (Figure 1.4) EPR parameters for different complexes show characteristic signatures with $A_{II}$ and $g_{II}$ values for N coordination ($g_{II} \sim 2.23$, $A_{II} \sim 170-190$ G) or N and O coordination ($g_{II} \sim 2.34$, $A_{II} \sim 130$G). The EPR parameters listed in Table 1.3 are intermediate between a tetrahedral ($A_{II} \sim 40-60$ G) and square pyramidal ($A_{II} \sim 160-200$ G) geometry around the copper ion and are characteristic for a mixed coordination environment of N and O ligands.

<table>
<thead>
<tr>
<th>Complex</th>
<th>$g_{II}$</th>
<th>$g_{\perp}$</th>
<th>$A_{II}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CukanA</td>
<td>2.427</td>
<td>2.104</td>
<td>110.35</td>
</tr>
<tr>
<td>CukanB</td>
<td>2.430</td>
<td>2.104</td>
<td>110.34</td>
</tr>
<tr>
<td>Cuneam</td>
<td>-</td>
<td>2.123</td>
<td>-</td>
</tr>
<tr>
<td>Cutobr</td>
<td>2.430</td>
<td>2.103</td>
<td>124.10</td>
</tr>
<tr>
<td>CuneoB</td>
<td>2.432</td>
<td>2.098</td>
<td>110.34</td>
</tr>
<tr>
<td>Cuparo</td>
<td>2.421</td>
<td>2.099</td>
<td>103.45</td>
</tr>
</tbody>
</table>

Table 1.3: EPR parameters for the isolated copper aminoglycosides measured at 20 K in frozen solution. The samples (5 mM) were prepared in 10 mM Tris-HCl pH 7.4 and frozen in an acetone/liquid N$_2$ bath.
Figure 1.4:  (A) Hyperfine features associated with copper (I = 3/2) binding to kanamycin A (1).  (B) X-band EPR of complexes 2-6. The samples (5 mM) were prepared in 10 mM Tris-HCl, pH 7.4 and frozen in an acetone/liquid N₂ bath. The spectra were collected at 20 K.
1.7 Conclusions

The isolated complexes could not be crystallized. The spectroscopic information obtained on the copper aminoglycoside complexes suggests that the species isolated is a 1:1 copper aminoglycoside complex. The uv-visible and EPR data are consistent with coordination to the Cu(II) is through the amine group and the vicinal hydroxyl group in case of kanamycin A, tobramycin and neamine. The other complexes do not show evidence for chelate formation from electronic spectroscopy or EPR.

1.8 Characterization of the copper aminoglycosides in solution

1.8.1 $^{13}$C- $T_1$ relaxation studies on the aminoglycosides in the presence of sub-stoichiometric concentrations of paramagnetic Cu$^{2+}$

In light of the information obtained from the characterization of the isolated complexes we decided to study them in solution. Since Cu(II) is paramagnetic it should influence the relaxation times of nuclei that are either in the vicinity of, or bound to it. $^{13}$C NMR measurements were carried out on a Bruker Avance-500 spectrometer working at 125.78 MHz at 298 K. Longitudinal relaxation times ($T_1$) were determined using an inversion recovery pulse sequence using various delay times ranging from 1ms to 10 s and minimum recycle times of 10 s (5 times $T_1$). Data for $^{13}$C measurements were processed using X-Win NMR version 2.1 software on a Silicon Graphics workstation. All NMR
samples were made in 100 mM HEPES, pH 7.3 in 10% D$_2$O except for kanamycin B which was done in Tris-HCl, pH 7.4. $^{13}$C assignments were obtained from previous studies.$^{12,17-19}$

Extensive $^{13}$C-NMR studies and $^1$H-NMR relaxation studies under basic pH conditions (9.5 or 10.2) have supported the binding of Cu$^{2+}$ to ring C of kanamycin A and ring A of neamine. Titration of various Cu$^{2+}$ concentrations (10-100 µM) into a constant concentration (100 mM) of aminoglycosides (neomycin B, kanamycin A, paromomycin, tobramycin, kanamycin B or neamine) provided substantial changes in the T$_1$ rates of select carbon atoms of the ligands. The T$_1$ relaxation rates of two carbon atoms in the backbone of kanamycin A, tobramycin and neamine were significantly affected in presence of 60 µM Cu$^{2+}$ concentrations. However, treatment with 150 µM Cu$^{2+}$ influenced the T$_1$ relaxation rates of only one carbon atom in the backbone of neomycin B and paromomycin (Table 1.5). Addition of further amounts of Cu$^{2+}$ caused line broadening in most of the carbon skeleton making it difficult to interpret the $^{13}$C spectrum. At pH 7.90, in kanamycin A, the C$_{3''}$-NH$_2$ carbons are influenced to a maximum extent (30 fold decrease in T$_1$), while considerable changes in T$_1$ values of C$_{4''}$-OH (~4 fold) are also seen. Usually a two-fold decrease in T$_1$ relaxation times indicates proximity of the paramagnetic metal ion to the nucleus of interest. Surprisingly, the other carbon atoms in the carbon skeleton of kanamycin A are either not influenced or show negligible change in their T$_1$ values. At pH 7.90 the metal center is more closely bound to the amine-N, than to the alkoxy oxygen, wherein the latter binds copper in a protonated
form. This influences the relaxation rates of the carbon atom bearing the amino group to a larger extent than the carbon atoms with alkoxy oxygen groups. Relaxation measurements were also performed at pH 5.40 and 9.75 in the presence of 60 µM Cu$^{2+}$. At pH 9.75, a five fold decrease in the $T_1$ of C$_{3''}$-NH$_2$ and a three fold decrease in $T_1$ of C$_{4''}$-OH was noticed. Under basic conditions, the carbon atoms, C$_{3''}$-NH$_2$ and C$_{4''}$-OH are equally affected, probably indicating that the metal center is equidistant from both binding sites. This would be possible, only if the alcoholic oxygen atom is deprotonated and binds in a C-O$^-$ fashion to the copper center. The latter conclusions are supported by $^{13}$C relaxation measurements carried out at pH 5.40 (wherein all the amines are protonated). Neither the C$_{3''}$-NH$_2$ nor C$_{4''}$-OH were greatly affected under these experimental conditions and weaker binding or rapid exchange was noticed for Cu$^{2+}$ binding to kanamycin A. Reactions of tobramycin with Cu$^{2+}$ (at pH 7.40) yielded similar results as seen with kanamycin A at pH 7.90 (Table 1.4), except that the magnitude of the decrease in $T_1$ was not as substantial. As found for kanamycin A, other carbon atoms in tobramycin are least affected upon addition of 60 µM Cu$^{2+}$ (Table 1.4). The similarity of the $T_1$ results with tobramycin and kanamycin A indicate that Cu$^{2+}$ binds to both these aminoglycosides by forming a chelate ring utilizing C$_{3''}$-NH$_2$ and C$_{4''}$-OH functional groups.
Figure 1.5: $^{13}$C-T$_1$ relaxation spectra of Cu$^{2+}$ (60 µm) and kanamycin A (100 mM) in 100 mM HEPES, pH 7.9. The delay times range from 10 s to 1 ms (A-H)
Table 1.4: $^{13}$C relaxation measurements of kanamycin A (A) and tobramycin (B) with 60 µM Cu(II). Data marked in bold indicate the largest change in relaxation times in the presence of Cu (II). Measurements were carried out in 100 mM HEPES, pH 7.4.
Longitudinal relaxation studies with neamine show maximum effects on the $T_1$ values of $C_2'$-NH$_2$ (30 fold decrease in $T_1$) upon Cu$^{2+}$ binding, while substantial effects on the $T_1$ of carbon atoms on $C_3'$-OH (~3 fold decrease), glycosidic $C_1'$-O (3 fold decrease) and $C_3'$-NH$_2$ (4 fold decrease) are also noticed. This is a more complex pattern than found for kanamycin A or tobramycin, but coordination of Cu$^{2+}$ to ring A has also been supported by proton $T_1$ relaxation measurements. From the $^{13}$C NMR data, we have been able to deduce the binding sites to ring A in neamine, (Table 1.5) without involving the glycosidic oxygen atom. The $C_1'$-O carbon showed a decrease (3 fold) in its $T_1$ times, however, $T_1$ of $C_4$ carbon is not influenced by presence of Cu$^{2+}$. Binding of the copper ion to the glycosidic oxygen should have influenced both carbon atoms to the same extent. These results support our view that the metal ion does not bind to the glycosidic oxygen atom, and forms a chelate utilizing $C_2'$-NH$_2$ and $C_3'$-OH. The relaxometric measurements using $^1$H-NMR by Mobashery and co-workers also refutes the involvement of $C_1'$-O and $C_3'$-NH$_2$ based on the paramagnetic contribution to $T_1$ ($T_1^e$) values.
Table 1.5: $^{13}$C relaxation measurements of neamine with 60 μM Cu(II). Data marked in bold suggests the largest change in relaxation times in the presence of Cu(II).

Measurements were carried out in 100 mM HEPES, pH 7.4.

Binding of copper to neomycin B (Table 1.6) and paromomycin (Table 1.7) was carried out under similar conditions. However, neither of these aminoglycosides showed substantial reactions with 60 μM Cu$^{2+}$. Upon increasing the concentrations to 150 μM Cu$^{2+}$, the NMR resonances corresponding to C2'-NH$_2$ in the A ring were broadened and
exhibited a two fold decrease in its T1 values. Paromomycin, on the other hand, did not show any changes in its 13C spectra under similar experimental conditions. Also, other carbon atoms in neomycin B bearing either an oxygen atom or an amino group are not influenced under these experimental conditions (Table 1.7). Surprisingly, we found a 4-fold decrease in carbon atom bound to glycosidic oxygen atom, C4 in ring B of neomycin. If the Cu2+ ion could chelate C2’-NH2 and C4-O atoms, then there should have been a direct influence on the C1'-O carbon atoms also. However, absence of any such effects refutes the above structure. The effect is probably due to induction of a nearby paramagnetic metal ion. We have synthesized and isolated a neomycin complex of copper, but found that the isolated material is unstable in solution over a period of 4h. Our data from 13C relaxation measurements indicates that Cu2+ is unable to form a chelate with neomycin B or paromomycin. Though Cu2+ can bind to one amino group in these two aminoglycosides, the complex is not stable due to the lack of a chelate ring, leading to rapid hydrolysis of the isolated copper-neomycin B complex.
### Table 1.6: $^{13}$C relaxation measurements of neomycin B with 150 µM Cu(II).

Measurements were carried out in 100 mM HEPES, pH 7.4, 10% D$_2$O.
Table 1.7: $^{13}$C relaxation measurements of paromomycin with 150 µM Cu(II). Data marked in bold suggests the largest change in relaxation times in the presence of Cu(II). Measurements were carried out in 100 mM HEPES, pH 7.4.
Kanamycin B on addition of 100 µM Cu$^{2+}$ showed moderate changes in T$_1$ relaxation times of all its carbon atoms in rings A and C. (Table 1.8) Carbon atoms in ring B (2-deoxystreptamine) remain unaffected as is evident from the T$_1$ data. Kanamycin B differs from kanamycin A in that it has a C$_2$-NH$_2$ group instead of C$_2$-OH. This additional amine group generates a potential chelating site that can now compete with the Cu$^{2+}$-binding site on ring C and thus it is not surprising that the relaxation times of all the carbon atoms on both rings (A and C) are affected to a similar extent. The atoms in ring A are affected to a slightly larger extent that those in ring C, implying that the Cu$^{2+}$ ions may predominantly reside close to ring A rather than C where unambiguous binding to ring C is established with the above studies for kanamycin A.
<table>
<thead>
<tr>
<th>C-Atom,</th>
<th>ppm</th>
<th>$T_1$, ms (kan B)</th>
<th>$T_1$, ms (kan B + Cu$^{2+}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-1&quot;, C</td>
<td>100.9</td>
<td>432.3</td>
<td>331.2</td>
</tr>
<tr>
<td>C-2&quot;, OH</td>
<td>69.6</td>
<td>399.08</td>
<td>323.2</td>
</tr>
<tr>
<td>C-3&quot;, NH$_2$</td>
<td>54.8</td>
<td>398.5</td>
<td>298.9</td>
</tr>
<tr>
<td>C-4&quot;, OH</td>
<td>66.6</td>
<td>395.6</td>
<td>272.5</td>
</tr>
<tr>
<td>C-5&quot;, C</td>
<td>70.5</td>
<td>402.2</td>
<td>280.2</td>
</tr>
<tr>
<td>C-6&quot;, OH</td>
<td>60.6</td>
<td>263.9</td>
<td>237.3</td>
</tr>
<tr>
<td>C-1&quot;, C</td>
<td>96.9</td>
<td>448.2</td>
<td>273.0</td>
</tr>
<tr>
<td>C-2&quot;, NH$_2$</td>
<td>55.8</td>
<td>358.2</td>
<td>228.5</td>
</tr>
<tr>
<td>C-3&quot;, OH</td>
<td>73.3</td>
<td>365.8</td>
<td>300.2</td>
</tr>
<tr>
<td>C-4&quot;, NH$_2$</td>
<td>69.3</td>
<td>423.9</td>
<td>268.0</td>
</tr>
<tr>
<td>C-5&quot;, NH$_2$</td>
<td>71.8</td>
<td>428.4</td>
<td>327.2</td>
</tr>
<tr>
<td>C-6&quot;, C</td>
<td>49.9</td>
<td>417.2</td>
<td>340.0</td>
</tr>
</tbody>
</table>

**Table 1.8:** $^{13}$C relaxation measurements of kanamycin B with 150 µM Cu(II).

Measurements were carried out in 100 mM HEPES, pH 7.4, 10% D$_2$O.
1.8.2 UV-visible spectroscopy to monitor $K_d$’s for Cu$^{2+}$ binding to aminoglycosides:

To a solution of 0.1 mM aminoglycoside was added 2 mM Cu$^{2+}$ solution in small ($\mu$L) increments and the charge transfer transitions for the respective complexes that are characteristic of chelate formation were monitored. The final aminoglycoside to Cu$^{2+}$ ratio was 1:8 (Figure 1.6). The data were fit to a one-site binding model to obtain the $K_d$ for the corresponding complexes (Table 1.9). Consistent with the NMR data copper binding to kanA and tobr monitored by uv-visible spectroscopy was found to be tight and in the micromolar range. For kanB and neoB Cu(II) binding was relatively weak and paromomycin gave a linear response. In the case of neamine the binding was not fast enough to be clearly monitored by charge transfer transitions in water. The synthesis as described elsewhere was carried out in methanol and requires heating for a longer period of time. The resulting isolated complex is very stable and does have a very strongly coordinated copper ion.
**Table 1.9:** $K_d$’s for aminoglycoside binding to Cu$^{2+}$ as monitored by charge transfer transitions. s in water were added in small increments to a solution of 0.1 mM aminoglycoside in water was added small aliquots of 2 mM Cu(II) stock solution to a final ratio of 8:1 (metal to ligand ratio). The monitoring wavelength for each aminoglycoside was selected from their observed CT bands in the isolated complexes.

<table>
<thead>
<tr>
<th>Aminoglycoside</th>
<th>$K_d$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kanamycin A</td>
<td>0.051</td>
</tr>
<tr>
<td>Kanamycin B</td>
<td>0.278</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>0.098</td>
</tr>
<tr>
<td>Neamine</td>
<td>1.367</td>
</tr>
<tr>
<td>Neomycin B</td>
<td>0.476</td>
</tr>
<tr>
<td>Paromomycin</td>
<td>NA</td>
</tr>
</tbody>
</table>
Figure 1.6: (A) Copper binding to kanamycin A, kanamycin B and tobramycin monitored using a charge transfer transition for each of the copper-aminoglycosides at 243, 250 and 245 nm respectively. (B) Copper binding to neomycin B, paromomycin and neamine monitored using a charge transfer transition for each of the copper-aminoglycosides at 250, 255 and 230 nm respectively.

2 mM Cu$^{2+}$ stock solutions in water were added in small increments to a solution of 0.1 mM aminoglycoside in water to a final ratio of 8:1, metal to ligand.
1.8.3 Conclusions:

The isolated copper complexes of aminoglycosides have been characterized by uv-vis, EPR and NMR spectroscopy. In spite of the absence of substantive evidence for Cu$^{2+}$ chelating to paromomycin, neomycin B and kanamycin B, we have successfully isolated the 1:1 (M: L) complexes of which the two former ones are indeed unstable in buffered solutions over a period of 4 h. CukanB rapidly hydrolyses in HEPES buffer, but is stable in Tris-HCl at pH 7.4. Cukan A and Cutobr form a stable chelate utilizing the amine and hydroxyl functionalities on Ring C whereas Cuneam does so, on ring A. Whether the Cuparo, CuneoB and CukanB form a chelate is not known, but the metal does anchor onto an amine group in one of the terminal rings. Thus Cu$^{2+}$ binding site on kanamycin A, neamine and tobramycin can be established with confidence with $T_1$ relaxation studies as against those for neomycin B, paromomycin and kanamycin B. The latter three antibiotics have a higher charge that may play a role in inefficient binding of Cu$^{2+}$ ions. In spite of the higher positive charge, tobramycin does show chelation to Cu$^{2+}$ ions which may be due to the absence of C$_3$·OH. The changes in $T_1$ values on addition of Cu$^{2+}$ to tobramycin are not as marked as in the case of kanamycin A which can be attributed to the increased electrostatic repulsion between the two (Cu$^{2+}$ and tobr). Further neomycin B does have a potential binding site on ring A, but may be hindered due to the presence of the ribose ring in close proximity. The pK$_a$’s of the amine groups must also play a critical role in the anchoring of the metal ion and it is not surprising that the amines with
pK\textsubscript{a}'s closer to physiological pH are preferred over others with higher pK\textsubscript{a}'s. In case of the 2-deoxystreptamine ring one of the amines have a pK\textsubscript{a} of ~ 5.6 which maybe attractive for metal ion binding but the presence of a positive charge arising from its adjacent C\textsubscript{1}-NH\textsubscript{3}\textsuperscript{+} may prevent it from doing so. The change in EPR from a free Cu\textsuperscript{2+} to a bound Cu\textsuperscript{2+} suggests there is a change in the coordination environment around the metal ion. The nature of such coordination is not clear from the above studies. Thus electrostatics, in addition to favorably located chelating sites, dictates the coordination site for copper binding to these antibiotics.
CHAPTER 2

MECHANISM OF OXIDATIVE DNA DAMAGE

2.1 Introduction

DNA cleavage has been studied extensively over the years by a number of researchers in order to characterize the binding site of a protein (foot printing with hydroxyl radicals), develop small molecules as nucleases, and as chemotherapeutics. Intercalation and minor or major groove binding are the typical modes of interaction of these small compounds with DNA. Closed circular DNA is relatively widespread in nature. The rapidly growing list of plasmid DNA includes those from mitochondrial DNA, the tumor viruses polyoma, SV40, and rabbit and human papilloma; certain bacteriophage DNA’s, the intracellular form of φ X174, M13 and λ; bacterial episomes; and the colicinogenic factor E1, a
bacterial plasmid.\textsuperscript{20-28} Studies of plasmid cleavage have therefore become very important the light of their presence in various infecting organisms. A drug that selectively eliminates a plasmid in the infecting organism over a normal DNA has tremendous potential for application as an antitumor, anti bacterial agent. A host of natural and artificial organic molecules are effective DNA cleavage agents that are based on radical cleavage chemistry in the presence of molecular oxygen. Flavin-oligonucleotides can carryout photocleavage of DNA by forming a triple helix with a double stranded DNA sequence of HIV-1.\textsuperscript{29} Anti tumor agents such as SR4233 are known to carryout DNA damage through hydroxyl radicals.\textsuperscript{30} Glycosylated molecules like neocarzinostatin show increased activity due to the sugar moiety. Neocarzinostatin is the first chromoprotein antitumour antibiotic to be characterized and is composed of a highly reactive aminoglycoside chromophore component bound covalently to a 113 amino acid carrier protein. It is a prototypical enediyne antitumor agent.\textsuperscript{31} Low levels of neocarzinostatin selectively inhibits DNA synthesis in sensitive bacterial and mammalian cells, induce degradation of existing DNA in bacteria, and produce breaks in DNA in mammalian cell lines.\textsuperscript{32-41}

The use of coordination complexes to promote DNA cleavage is routine and very effective. These are usually based on redox chemistry of the metal center that eventually generates radical species which carry out chemistry on the ribose ring eventually resulting in strand scission. Copper, iron, nickel, cobalt, zinc, ruthenium, rhodium, iridium, platinum etc are the metals of choice, although lanthanides are also studied in
this respect. Complexes of copper are predominant in this application. Metal complexes are especially important and heavily used as effective nucleases e.g. Co-salen complexes carryout DNA damage in the presence of molecular oxygen or dithiothreitol. Iron (II) triazacyclononane derivatives carry out efficient DNA cleavage in the presence of reducing agents at physiological conditions.

The cleavage activity is improved ten times (in terms of concentration of reagent used) on attachment of psoralen (a DNA cross linking agent). Iron (II) ethylenediaminetetraacetic acid (EDTA) is used for chemical footprinting, with hydrogen peroxide as the co-reactant, with little or no specificity. When tethered to methidium, distamycin or oligonucleotides this complex has been used to promote efficient double strand cleavage of DNA. Iron (II)-bleomycin (BLM) degradation of DNA is sequence selective and results in preferential cleavage at certain GT and GC sequences and takes place in the presence of oxygen. Co (III) and Mn(II)/ Mn (III) complexes of BLM have also been shown to do chemistry on DNA. Cu (II)-BLM retards growth of tumor cells in tissue cultures and tumor growth in animals but does not show in vitro DNA damage. Complexes of rhodium and ruthenium with bipyridyl ligands, recognize DNA base mismatches and carryout cleavage on photoactivation. Phenenthroline complexes of rhodium exhibit very little selectivity for base mismatches as against the [Ru(bpy)_{2}(chrysi)]^{3+} complex which does so very selectively. Cr (V)- 2-ethyl-2-hydroxybutyric acid complex, [CrO(ehba)_{2}] does oxidative DNA damage in the presence of hydrogen peroxide.
Oxidative degradation of DNA has been demonstrated to follow a number of pathways, depending on the cleavage agent. Proton abstraction from one of the C-1’, C-2’, C-3’, C-4’ or C-5’ ribose carbons is followed by a series of elimination reactions that afford a variety of small molecule products characteristic of the specific cleavage pathway. Bleomycin mediated cleavage shows evidence of both a major C-4’H cleavage path and for C-1’H abstraction. While copper phenanthroline and copper desferal had previously been assigned a major C-1’ H cleavage path, more recent work has indicated that C-4’H and C-5’H abstractions are the only routes that lead to backbone scission. Enediynes, oxo chromium reagents and manganese porphyrin derivatives have also been shown to mediate C-1’ H, C-4’ H, and C-5’ H abstraction. An example of C-2’ H abstraction has been reported for 5-iodouracil. With the exception of Barton’s rhodium complexes, which mediate photocleavage via C-3’ H abstraction, most cleavage agents typically yield a mixture of products, indicative of relatively non-specific cleavage pathways.

Sreedhara et al have demonstrated CukanA and Cuneam to be highly efficient nucelases for cleavage of both RNA and plasmid DNA under physiological conditions. Either can mediate oxidative or hydrolytic cleavage of RNA and DNA. Hydrolytic cleavage of plasmid DNA exhibits multi-turnover kinetics, with Michaelis-Menten behavior, and a catalytic rate enhancement of ~ 10^8-fold relative to uncatalyzed hydrolysis was observed. CukanA mediates an efficient and unusual linearization of plasmid DNA directly without going through the nicked form of DNA. CukanA and Cuneam were both
seen to cleave the Rev response element in a similar fashion in the presence or absence of co-reactant (hydrolytic and oxidative conditions).\textsuperscript{79}

Copper aminoglycosides show great promise as chemical nucleases and the following section describes the mechanism of oxidative damage of plasmid DNA on a molecular level.

### 2.2 Electron Paramagnetic Resonance Spectroscopy

We were interested in trapping reactive intermediates, radicals or others from the reactions of copper aminoglycosides and either hydrogen peroxide or ascorbate in the presence or absence of nucleic acid substrate. Rapid freeze quench in the millisecond (ms) range followed by EPR of the frozen sample would potentially trap the reactive intermediates. Experiments were carried out with this rapid freeze quench technique and reinforced by room temperature studies of the radical species.

#### 2.2.1 Experimental.

Rapid freeze quench was done with a rapid freeze quench system from Update Instruments, Inc. with incubation times of 6, 10, 16, 100-700 ms (every 100 ms). The solutions were loaded into two syringes and reacted for the specified time in a mixing chamber followed by rapid freezing in a bath at -150\textdegree C (isopentane and liquid nitrogen).
For the reactions in the presence of substrate, the substrate and CukanA were mixed together in one syringe and the other syringe was filled with the co-reactant.

For slow quench experiments the solutions were mixed manually for the said incubation times (10 s or 20 s) and frozen in an acetone/ liquid N\textsubscript{2} freezing bath. Spin integration of the signals was done using copper perchlorate as the standard. The software used was Simfonia. EPR of the frozen samples was taken predominantly at 20 K unless mentioned otherwise.

The room temperature trapping of hydroxyl radicals with dimethyl pyrroline oxide (DMPO) was carried out in a flat cell and the mixing was done under argon to prevent degradation of the DMPO.

2.2.2 Results and Discussion:

EPR parameters are distinctly different for different classes of Cu (II) complexes. A single unpaired electron gives a signal at $g \sim 2.0043$. Cu\textsuperscript{2+} has a nuclear spin of $\frac{3}{2}$, which splits the signal into four ($2nI+1= 2 \times 1 \times \frac{3}{2} + 1 = 4$) at lower temperatures. In the case of Cu(II) EPR, $A_{II}$ values are indicative of the geometry around the metal ion. The values of $A_{II}$ increase on N-ligation, decrease for O donors and are the lowest for S-donors. Distorted tetrahedral or tetragonal geometries give values between 50-90 G. CukanA by itself has $A_{II}$ around 110 G, which suggests more of an octahedral to tetragonal coordination as expected. The EPR spectra of Cu-aminoglycosides suggest it
to adopt a tetragonal geometry with two coordination sites occupied by the aminoglycoside and the rest by water molecules.

![EPR spectrum graph](image.png)

**Figure 2.1:** X-band EPR spectrum of CukanA (blue) at 20 K in the presence of p(dA)-p(dT) (red), and p(A)-p(U) (green). On addition of the nucleic acid substrate the geometry around the Cu$^{2+}$ changes to square pyramidal $A_{II} = 172$ G, for DNA and 176 G for RNA.

Addition of substrate (p(dA)-p(dT) or p(A)-p(U)) to CukanA changed the EPR signal substantially, indicating it’s binding to the substrate. **(Figure 2.1)** The change in EPR parameters is indicative of a change in geometry around the Cu$^{2+}$ center **(Figure 2.1).**
CukanA carries out hydrolysis of plasmid DNA very efficiently at micromolar concentrations. Binding in this case precedes chemistry and it is not surprising that the Cu$^{2+}$ EPR shows such marked changes on the addition of substrate.

**Figure 2.2:** (A) EPR spectrum of a freeze trapped solution of CukanA (green) and CukanA + H$_2$O$_2$ (1:2) (red) 16 msec. (B) CukanA + Ascorbate (1:2) (red), 16 msec.

Hydrogen peroxide on addition to CukanA (2:1) changed the $A_H$ values from a tetragonal ($\sim$110 G) system to a square pyramidal ($\sim$185 G) geometry. An additional feature on the perpendicular signal is seen which suggests that an additional ligand must bind the Cu$^{2+}$
center along the z-axis. In case of the ascorbate reactions the same feature on the perpendicular signal is observed in addition to a sharp signal at $g \sim 2.090$ which may correspond to the ascorbate radical. (Figure 2.2)

In both cases, peroxide and ascorbate, the signal due to $\text{Cu}^{2+}$ reduces in intensity for shorter incubation times (< 100 ms) followed by an increase for longer incubations (<700 ms). This suggests that the paramagnetic $\text{Cu}^{2+}$ center gets converted to a diamagnetic center in the presence of a co reactant. Power saturation and variable temperature experiments on these samples change the intensity of all the signals to the same extent suggesting the presence of a single species in solution. These features are preserved in the presence of substrate. The additional feature on the perpendicular signal suggests a bound radical, which splits the $g_{\perp}$ signal into two. The Cu(III) species generated by peroxide oxidation may be reduced to a Cu(II)- oxene species that gives rise to the observed EPR signal. The peroxide can generate hydroxyl radicals in the presence of Cu(II) can produce superoxide radicals, which on dismutation can generate peroxyl or hydroxyl radicals. We have observed that superoxide scavengers completely inhibit the cleavage reaction in the presence of ascorbate.\textsuperscript{76} Thus the presence of reactive oxygen species in addition to hydroxyl radicals cannot be ruled out as active intermediates in the oxidative reactions of DNA cleavage.

Room temperature spin trapping experiments with dimethyl pyroline oxide (DMPO) gives a signature spectrum for trapped hydroxyl radicals (Figure 2.3). The presence of hydroxyl radicals was previously established with rhodamine B by means of a
The reactions with ascorbate did not give the signature spectrum suggesting the radical maybe copper based even in the absence of substrate (DNA) or the intermediate formed is a different one.

The reactions with ascorbate generate hydrogen peroxide, which in turn reacts with the \( \text{Cu}^+ \) center to generate hydroxyl radicals. This hydroxyl radical maybe coupled to the \( \text{Cu}^{2+} \) center and hence does not show up in the trapping experiment. The hydrogen peroxide reaction is a direct method to generate radical species and is hence faster and more clear-cut.

Spin integration of the signals following reaction of CukanA and the co-reactant at 10 s and 20 s shows a drop in the copper signal for the first 10 s followed by an increase at 20 s. The total spin of the system equilibrates to a final value where the paramagnetic and diamagnetic species are in equilibrium. Copper perchlorate was used as the standard to measure spin density of the signal for a specific concentration. Depending on the molar excess of ascorbate or peroxide used for the reaction the total paramagnetic spin was observed to change relative to the initial incubation. A higher molar excess of the co-reactant converted more of the paramagnetic spin to diamagnetic. The ascorbate reduces the \( \text{Cu}^{2+} \) center to \( \text{Cu}^+ \) and back, whereas the peroxide may oxidize the \( \text{Cu}^{2+} \) to \( \text{Cu}^{3+} \) and back.
Figure 2.3: The green spectrum represents the EPR of a solution of CukanA and H₂O₂ mixed in a ratio of 1:2 in the presence of DMPO. The brown spectrum is that of CukanA. The EPR are measured in solution at room temperature (under isotropic conditions). Inset shows the signature of trapped hydroxyl radicals by DMPO.
2.2.3 Conclusions

The EPR data suggests that the reactive hydroxyl radicals generated during the reaction of CukanA or Cuneam with co-reactant peroxide are predominantly copper based, leading to substantial changes in the EPR of the Cu\(^{2+}\) center. The co-reactant carries out redox chemistry at the copper center to generate a diamagnetic species, which is recycled back and forth during the course of the reaction. The presence of a superoxide or ascorbate radical cannot be ruled out, although they were not observed by EPR.

2.3 Mechanism of Oxidative Damage to Plasmid DNA

2.3.1 Experimental

(i) HPLC Analysis.

In a typical reaction, 100 µg of plasmid DNA (pT7-7) was treated with 0.5 mM of 1 or 2 and a ten-fold molar excess of H\(_2\)O\(_2\) or ascorbate in 10 mM HEPES (or Tris-HCl), pH 7.4 at 37\(^\circ\)C. After incubation for an hour the reaction mixture was loaded onto a Pharmacia Biotech C-18 Sephasil Peptide (5 µM, ST 4.6/250) reverse phase column equilibrated with 0.1 M NH\(_4\)OAc, pH 6.8 (solvent system A). Products were eluted with a gradient
profile (0-5 min, 100% A; 5-30 min, 0-2% B in A; 30-40 min, 2-10% B in A; 40-50 min, 10% B in A; 50-60 min, 10-25% B in A) where system B was acetonitrile. The same reaction mixture was heated at 85°C for 20 min, loaded onto a reverse phase column, and the elution profile was recorded. Independent control runs using authentic materials confirmed the identity of the products of the cleavage reaction. Known concentrations of bases (Sigma Chem. Co) were loaded on to the reverse phase column and the corresponding peak areas were measured. A calibration plot of peak areas versus concentration was used to calculate the concentration of bases released during cleavage reactions. 5-MF was isolated and characterized by mass spectrometry (base peak at 96 amu) and UV-vis spectroscopy (with $\lambda_{\text{max}} \sim 260$ nm).

(ii) Thiobarbituric acid assay:

The standard thiobarbituric acid (TBA) assay was carried out by heating (95°C) the reaction mixture in 0.6% TBA for 20 min. The resulting pink colored solution corresponding to the adduct of TBA and malondialdehyde exhibited a $\lambda_{\text{max}}$ at 532 nm. A calibration curve was generated using 1 – 10 $\mu$M malondialdehyde as a standard.

(iii) Quantitation of the C-1’ H pathway:

The HPLC peak corresponding to 5-MF was collected and its absorbance measured. The concentration of 5-MF was obtained by using the published extinction coefficient $\varepsilon = 1400 \text{ M}^{-1}\text{cm}^{-1}$. For every mole of base released, 1 mole of 5-MF is generated by the
C-1’ H abstraction. The concentration of base responsible for the C-1’ H pathway was therefore determined.

2.3.2 Highly specific plasmid DNA damage.

The HPLC profile for CukanA cleaved plasmid DNA (Figure 2.4A) shows the release of cytosine (C), guanine (G), thymine (T), and adenine (A) following residual DNA and larger product fragments. An additional peak corresponding to 5-methylene furanone (5-MF) (Figure 2.4B) appears when the reaction mixture is heated at 85°C for 20 min following incubation.
Figure 2.4: HPLC traces of reaction mixture with 100 mg plasmid, 0.5 mM CukanA, 5 mM H₂O₂ incubated at 37°C, (A) without heating at 95°C; (B) after heating at 95°C for 10 min. The bases are released in the order seen, C, G, T and A. The additional peak for 5MF is seen only on heating.
C-1’ H abstraction generates a C-1’ radical that is further oxidized to a carbocation and forms either a 2’-deoxyribonolactone\textsuperscript{69} or a 1’,2’- dehydronucleotide intermediate\textsuperscript{71} after subsequent attack by H$_2$O. Both intermediates are stable at pH 7.4 and 37ºC, the conditions of our cleavage reaction, and the HPLC elution profile corroborates heat treatment to be a prerequisite for the release of 5-MF (\textbf{Figure 2.4}). Thus C-1’ H abstraction leads to the formation of abasic sites with no apparent strand cleavage. C-4’ H abstraction yields distinct reaction products, depending on the attacking species following formation of the radical intermediate. In the presence of dioxygen, base propenals (detected by the TBA assay) (\textbf{Figure 2.5}) and 3’- phosphoglycolate termini are generated (\textbf{Figure 2.6}; products (C) and (D), respectively). When water is the attacking species the pathway again does not give rise to direct strand cleavage, but base release is observed. Under anaerobic conditions the TBA assay was negative, indicating the absence of a C-4’ H (O$_2$) path as expected.

None of the expected products for C-2’ H, C-3’ H and C-5’ H degradation paths were observed (even after alkaline treatment),\textsuperscript{35,73,75} consistent with C-4’ H abstraction path as the only route leading to plasmid cleavage.
Figure 2.5: Thiobarbituric acid assay for 100 µg of plasmid DNA (pUC19) + 0.5 mM CukanA + 5 mM ascorbate, A; + 5 mM H₂O₂, B
Figure 2.6: C-1’ H abstraction leads to the formation of 2’-deoxyribonolactone if attacked by hydroxyl radical or α1’, 2’-dehydronucleotide intermediate. Upon further eliminations at higher temperatures these result in strand scission.
A C-1' H path was observed only in the case of supercoiled plasmid with peroxide as co reactant and the fraction of C-1’ H abstraction was never found to be more than 12% of the C-4’ H path, based on quantitation of the 5-MF released. Reaction with ascorbate/O₂ appears to proceed via an intermediate copper species that does not mediate C-1’ H abstraction. Neither linear plasmid, nor general polynucleotide duplex DNA (discussed below) show evidence for C-1’ H loss, whereas C-4’ H chemistry is observed with both substrates, and also using either H₂O₂ or ascorbate/O₂ as co reactants. This difference most likely reflects structural perturbations of the supercoiled strands that make the C-1’ H accessible for abstraction.

2.4 Reactivity profiles for polynucleotide duplexes p(dA)-p(dT) and p(dG)-p(dC)

2.4.1 Major pathway of cleavage.

The reactivity of CukanA and Cuneam toward two DNA duplexes, p(dA)-p(dT) and p(dG)-p(dC), was also examined in the presence of either hydrogen peroxide or ascorbate, under reaction conditions that were similar to those used for plasmid DNA. Base release was observed and quantitated by HPLC analysis. The occurrence of base propenals was confirmed with the thiobarbituric acid assay. Figure (2.7) shows the
presence of base propenals in the reactions of p(dA)-p(dT) treated with the respective reagents.

![Graph showing absorption units vs wavelength for different treatments](image)

**Figure 2.7:** Thiobarbituric acid assay for reactions with p(dA)-p(dT). A solution of 50 µg of duplex in 10 mM HEPES, pH 7.4, was treated with either 0.5 mM CukanA or Cuneam and 5 mM peroxide or ascorbate for 2 h at 37°C followed by the addition of 400 µL of 0.6% TBA and boiling at 95°C for 20 min.
HPLC analyses of the duplexes under similar conditions as the plasmid resulted in base release, which was also quantitated using calibration curves for nucleotide bases and the TBA assay. Reactions with p(dA)-p(dT) were more productive than p(dG)-p(dC) with both peroxide and ascorbate with the former resulting in higher cleavage than ascorbate. Under hydrolytic conditions, in the absence of added H₂O₂ or ascorbate, treatment of DNA with either 0.5 mM CuSO₄ or 0.5 mM aminoglycoside, under otherwise similar reaction conditions, released no free base, or 5-MF, or other standard oxidative degradation products were observed for either duplex or plasmid DNA. Reaction of CuSO₄ and a ten-fold excess of either H₂O₂ or ascorbate, again all other conditions being maintained, showed minimal (<10 %) base release, as compared to treatment with either 1 or 2, but without the production of base propenals. The latter observation suggests an alternative path for production of nucleotide base. With increasing amounts of free Cu²⁺ (maintaining a ten times excess of ascorbate or H₂O₂) the concentration of released bases increased. However, base release was found to be random with free Cu²⁺ and co-reactant, and did not show any preference for either pyrimidines or purines, consistent with a distinct reaction path. No reaction was observed with H₂O₂ or ascorbate (with or without added neamine or kanamycin A) in the absence of Cu²⁺(aq), CukanA or Cuneam.

In contrast to other DNA cleavage agents that follow an oxidative path (cited earlier), copper aminoglycosides (CukanA and Cuneam) follow a highly specific route mediated by abstraction of the C-4’ H. Close proximity of C-4’ H’s on opposing strands separated by two additional base-paired nucleotides also provides a rational explanation for the
observed linearization of plasmid (or double-strand cleavage of duplex DNA). A reactive copper moiety placed in the minor groove would be well placed to execute a double-strand scission with minor structural rearrangement between cleavage reactions.

2.4.2 Netropsin inhibition of DNA cleavage:

The C-4’ H’s are placed in the minor groove of the DNA and any reagent that selectively carries out cleavage or H abstraction at this carbon is expected to bind in the minor groove. Figure 2.8 shows the orientation of the H atoms in the minor and major grooves of DNA. To confirm our hypothesis we used two minor groove-binding agents, netropsin and Hoechst 33258 to locate the binding site for the complexes. For supercoiled plasmid the minor and major grooves are not well defined whereas for the homopolymers mentioned above the grooves are very much in place and a minor groove-binding molecule should compete with CukanA and Cuneam efficiently to inhibit cleavage. Of the two, the latter has a higher binding affinity and was not very useful since very low concentrations of it precipitated the DNA.
**Figure 2.8:** Orientation of the H atoms in the major and minor groove of DNA. The H atoms represented by the wedges point into the minor groove. $C-1'$, $C-4'$, $C-5'$ are secondary carbon atoms and are more susceptible to abstraction by a nearby radical.

Figure reproduced from reference. 81
Figure 2.9: Netropsin inhibition of the homopolymeric duplexes in the presence of CukanA and hydrogen peroxide. The reactions were incubated with increasing concentrations of netropsin (0-1.0 mM) in the presence of CukanA (0.5 mM) and H₂O₂ (5 mM) for 2 h at 37°C followed by treatment of 0.6% TBA at 95°C for 20 min. Inset shows the uv-vis response for the p(dA)-p(dT) duplex in CukanA / peroxide.

Netropsin on the other hand has a slightly lower binding affinity and was appropriate for the purpose. Netropsin inhibition was studied with the thiobarbituric acid assay for both
duplexes and plasmid as well. The plasmid did not show inhibition in the presence of netropsin due to its supercoiled structure. Alternatively p(dA)-p(dT) and p(dG)-p(dC) show substantial inhibition. (Figure 2.9) shows the inhibition profile for both duplexes. p(dG)-p(dC) does not show a very good response to the TBA assay as compared to p(dA)-p(dT). This maybe due to its structure that is not a B-form DNA structure.

2.5 Quantitation of cleavage for duplexes and plasmid (pUC19)

Quantitation of the ratio of C-4’ H (O₂) to C-4’ H (H₂O) paths revealed a preference for the C-4’ H (O₂) path, both for the polynucleotide duplexes (74% versus 26%), and for a plasmid DNA (59% versus 41%). The factors that favor one path over another are not clear, and the lack of a clear preference for one path over another has previously been observed for Cu (phen)₂⁺ cleavage of plasmid and duplex sequences. Base release following peroxide mediated cleavage of either plasmid or duplex DNA by CukanA or Cuneam also shows no obvious trend, other than a variable tendency for release of purine over pyrimidine.
Figure 2.10: (A) Calibration plots for the four DNA bases A, G, T and C obtained from plotting peak areas versus concentration (mM). (B) Beer’s law graph for the TBA assay obtained by using malondialdehyde as standard.

Base release before and after heating was quantitated using the base calibration plots shown in Figure 2.10A. For every 1 mole of 5-MF released, 1 mole of base is released by the C-1’H pathway. Total base release was quantitated from the analyses of the peak areas of the bases after heating the sample. Base release resulting from the C-1’H
pathway was subtracted to obtain the base release due to C-4’H abstraction. The C-4’H pathway can be further divided into two sub-pathways depending on the attacking species (H₂O or O₂). The O₂ pathway was quantitated using the TBA assay and this was then deducted from the total C-4’H pathway to give the cleavage due to C-4’H / H₂O attack. The cleavage via the C-1’ H abstraction is 4% of the total cleavage. Of the remaining 96%, 73% goes through an O₂ attack on the C-4’ radical whereas 23% is due to attack of water on the same. Plasmid cleavage in the presence of peroxide or ascorbate was comparable. The above percentages are for cleavage of plasmid with CukanA in the presence of ascorbate.

For the duplexes, the C-1’H abstraction pathway was not observed and all of the cleavage was via C-4’H abstraction followed by attack by a water molecule or oxygen. The pathway which proceeds by H₂O attack on the C-4’ radical by H₂O resulting in base release amounts to 35% of the total cleavage. The alternative pathway proceeds via O₂ attack on the C-4’ radical to give base propenals which were quantitated by the TBA assay.

2.6 Conclusions

In the case of oxidative damage to DNA the mechanism is highly specific and occurs via C-4’ H abstraction. This is novel and in turn leads to efficient double strand cleavage. The copper aminoglycosides bind in the minor groove of double stranded DNA, which is
established by inhibition in the presence of netropsin. The reactions are oxygen mediated and cleavage is completely inhibited in the absence of oxygen when ascorbate is the co-reactant. Hydroxyl radicals abstract a H-atom from the C-4’ position to give a C-4’ radical, which undergoes attack by either oxygen or water to yield the reaction products. Base release is observed and there is no apparent selectivity for any nucleotide base by the CukanA or Cuneam. There is a change in the geometry around the Cu(II) center on addition of nucleic acid implying binding of the same to the RNA or DNA substrate. Thus reactions of cleavage are controlled and binding is required to orient the Cu(II) moiety for cleavage. Thus CukanA and Cuneam show potential as highly specific chemical nucleases and can be further studied to check their activity in vivo.
CHAPTER 3

ROLE OF METAL IONS IN THE CATALYSIS OF THE
HAMMERHEAD RIBOZYME

3.1 Introduction

Hammerhead ribozymes are one of the most studied active RNA motifs that carry out phosphodiester cleavage in the presence of divalent metal ions. The hammerhead comprises an inner conserved core that is absolutely essential for activity (Figure 3.1). These residues are involved in formation of key structural motifs and divalent metal ion binding sites.
**Figure 3.1:** Hammerhead ribozyme (34 mer) and the 5’ fluorescein labeled substrate (13 mer). The nucleotides in bold letters are the conserved residues and the cleavage site (between C17 and G1.1) is marked with an arrow.

The mechanism of the highly specific cleavage (between residues 17 and 1.1) is an in-line attack by the 2’-OH on the 3’-phosphate resulting in a 5’-OH terminus in addition to a 2’,3- cyclic phosphate. This cleavage reaction is catalyzed by the presence of divalent metal ions, with Mg$^{2+}$ as the metal ion of choice *in vivo* due to its high charge density and physiological availability. However hammerhead is also activated by other divalent
metal ions with the order of cleavage rates as follows $\text{Mn}^{2+} > \text{Mg}^{2+} > \text{Co}^{2+} > \text{Ca}^{2+} > \text{Sr}^{2+} > \text{Ba}^{2+} > \text{Cd}^{2+} > \text{Zn}^{2+} > \text{Pb}^{2+}$. Background cleavage rates were also observed in the presence of 1 mM EDTA and 0.5 mM spermine, which suggest that the hammerhead exhibits cleavage if the structure is stabilized by other means.\textsuperscript{82} NMR has demonstrated the formation of the 3 stems of the hammerhead in the absence of added $\text{Mg}^{2+}$ ions.\textsuperscript{83} A study of relative affinities and activities of divalent ions in hammerhead ribozymes reveals two sets of divalent binding sites for $\text{Mg}^{2+}$, but just one set for the other cations $\text{Cd}^{2+}$, $\text{Co}^{2+}$ and $\text{Zn}^{2+}$.\textsuperscript{84} The latter three ions have a higher affinity for the ribozyme as compared to $\text{Mg}^{2+}$. The relative activities follow the same trend as the affinities except for $\text{Zn}^{2+}$, which does not exhibit any activity. Alternatively the highest concentration of metal required for maximal rates does not correlate with the affinities.\textsuperscript{84} Molar concentrations of monovalent ions such as $\text{Li}^+$, $\text{Na}^+$ etc also promote site-specific cleavage, but is thought to do so by a different mechanism since the substitutions that affect divalent metal ion supported cleavage are not critical for the monovalent case.\textsuperscript{85} The role of the metal ion has been extensively debated over the years. A linear response of the rate to increasing pH with a slope of unity has been used to suggest a metal hydroxide species.\textsuperscript{86} The lower the $\text{pK}_a$ of the water bound to the metal the faster the reaction catalyzed by that metal. The deuterium isotope effect (4.4) observed is seen as a consequence of the lower concentration of the $\text{M}^{2+}$-OD as compared to $\text{M}^{2+}$-OH species.\textsuperscript{87} $\text{Mn}^{2+}$ catalyzed hammerhead cleavage exhibits a higher rate constant than that for $\text{Mg}^{2+}$. The higher rate for $\text{Mn}^{2+}$ has been explained in terms of the higher
concentration of the Mn\(^{2+}\)-OH species in solution as compared to Mg\(^{2+}\)-OH at a given pH, taking into account the pK\(_a\) of the bound water. The rate of the Mn\(^{2+}\) catalyzed reaction is 14 times greater than Mg\(^{2+}\) and is explained by the two metal ion mechanism. The concentration of the [Mn\(^{2+}\)-OH] (6.3 times higher than [Mg\(^{2+}\)-OH] species) is recognized as the sole contributing factor to the difference in the rate and is explained in terms of two metal ions carrying out cleavage. One of the metals ions (as M\(^{2+}\)-OH) is proposed to activate the 2’-OH for attack on the phosphate whereas the other stabilizes the leaving group.\(^{88}\) Taira and coworkers have carried out molecular orbital calculations and have concluded that the cleavage of the 5’-phosphate is the rate-determining step. The greater stabilization by the Mn\(^{2+}\) of the leaving group as compared to Mg\(^{2+}\) results in the higher rate.\(^{89}\) The substitution of the bridging –O- to –S- at the cleavage site does not reproduce the kinetic ratio mentioned above and lead Kuimelis et al to refute the presence of the second metal ion.\(^{90}\) Zhou et al explain this effect by invoking the two metal ion mechanism in case of the wild type ribozyme whereas a one metal ion model for the thio bridged ribozyme wherein it is argued that the P-S bond is weak to begin with and hence does not need additional stabilization for cleavage.\(^{88}\) Crystal structures of an RNA/DNA hybrid or an all RNA hybrid with a 2’-OMe modification at the cleavage site, show the same overall arrangements of the three stems and the characteristic uridine turn revealing multiple binding sites for divalent ions.\(^{91, 92}\) Hammerhead crystals soaked in a solution of divalent ions reveal a recurring divalent metal ion-binding site A9/G10.1. A Mg\(^{2+}\) ion is seen to bind via a water molecule to the
N7 of G, and directly to the pro S phosphate of A9. Pley and coworkers see the pro Rp phosphate and the N7 of G10.1 bound directly to the Mn$^{2+}$. Scott et al crystallized the hammerhead at relatively low ionic strength and these crystals show 5 metal ion binding sites after soaking in a solution of Mg$^{2+}$ or Mn$^{2+}$. Of the five sites, the A9/G10.1 site is proposed to bind Mg$^{2+}$ as a pentaaquo Mg(II) species (one direct metal to phosphate bond) whereas all other sites show Mg$^{2+}$ interacting via bound water molecules (outer sphere interactions). A metal ion bound to the G5 residue is also observed and is thought to play a role in the catalysis. The crystal structures of hammerhead are said to be in the ground state since the scissile phosphate is not oriented for attack by the 2’-OH. A freeze-trapped intermediate indicates movement of the scissile phosphate when the crystals were poised at a higher pH 8.5 in the presence of Co$^{2+}$. An additional metal ion-binding site in the structure compatible with the in-line attack was also observed. The presence of a 2’-OH is essential to see the conformational change leading to the movement of the scissile phosphate.

$^{31}$P-NMR and molecular modeling studies have identified the phosphate of A13 as a high affinity binding site with an apparent dissociation constant of 250 - 570 µM. Tanaka and coworkers observed the presence of tandem G.A base pairs flanked on either side by G.C base pairs as sufficient to capture metal ions such as Mg$^{2+}$ and Cd$^{2+}$ using $^1$H, $^{13}$C and $^{31}$P NMR spectroscopy. This particular motif binds metal ions even in the absence of other structural elements of the hammerhead ribozyme.
Phosphorothioate substitutions at the pro-R phosphate of A9/G10.1 (RpS) site and the cleavage site C17 show reduction in hammerhead activity in the presence of divalent ions like Mg\textsuperscript{2+}, which is referred to as the thio effect. This thio effect rescued by thiophillic metals like Cd\textsuperscript{2+} has been taken as proof for the metal ion binding to these substituted residues. Direct Cd\textsuperscript{2+} binding with an affinity of 25 µM has been proposed for the RpS substitution at the A9/G10.1 site.\textsuperscript{99} Similarly the RpS substitution at the cleavage site phosphate can be rescued by Cd\textsuperscript{2+} in the presence of background divalent ions (50 mM Mg\textsuperscript{2+} or 100 mM Ca\textsuperscript{2+}), but direct metal binding to this residue has been refuted. The metal ion (Cd\textsuperscript{2+}) has similar affinities for all three substrates wild type, RpS and SpS (at cleavage site), which would suggest an alternate binding site (A9/G10.1) for the metal ion. A common activated complex has been suggested for Cd\textsuperscript{2+} catalyzed rate. The activated complex thus would include binding of the Cd\textsuperscript{2+} to the A9/G10.1 site as established by Peracchi et al and lead to rescue of cleavage activity. The rate of cleavage in the presence of Cd\textsuperscript{2+} alone is less than Mg\textsuperscript{2+} and Mn\textsuperscript{2+}.\textsuperscript{100, 101} \textsuperscript{31}P-NMR in the presence of Cd\textsuperscript{2+} suggests that the metal ion interacts directly and with high affinity to the RpS and SpS substitutions at the A9/G10.1 site but not with similar substitutions at the cleavage site. The addition of Cd\textsuperscript{2+} to the latter leads to small changes in the chemical shift of the \textsuperscript{31}P that are stereo specific as far as Cd\textsuperscript{2+} binding is concerned.\textsuperscript{102} The thio effect seen on substitutions of pro R phosphates of A13 and A14 cannot be rescued by the addition of Cd\textsuperscript{2+}, suggesting a different mechanism for loss of activity.\textsuperscript{103} If the Cd\textsuperscript{2+} does not play a visible role in rescue; why then is there a thio-effect to begin with?
Horton et al have utilized Electron Paramagnetic Resonance Spectroscopy to measure the affinity of Mn$^{2+}$ for the hammerhead ribozyme. Two sets of sites were observed for an RNA-RNA hybrid with a 2’-OMe substitution at the cleavage site with $n_1 = 2.5 \pm 0.9$, $K_{d(1)} = 3.3 \pm 2.7 \mu M$, and $n_2 = 7.1 \pm 0.7$, $K_{d(2)} = 122 \pm 12.8 \mu M$ in 0.1 M NaCl.\textsuperscript{104} The model used considers the metal ion binding sites to be non-interacting. The authors report the high affinity-binding site as the A9/G10.1 site observed previously in crystal structures as well as from phosphorothioate experiments.\textsuperscript{105} Addition of cobalt hexaammine leads to loss in hammerhead activity, which is concurrent with loss of bound Mn$^{2+}$ ions. The apparent binding affinity of [Co (NH$_3$)$_6$]$^{3+}$ in the presence of 0.5 mM Mn$^{2+}$ and 0.1 M NaCl was obtained as $22 \pm 4.2 \mu M$.\textsuperscript{106} The authors consider this as inhibition of hammerhead cleavage by [Co(NH$_3$)$_6$]$^{3+}$ similar to the inhibition seen with lanthanide ions the difference being, binding of trivalent lanthanides to the G5, leads to complete inhibition of hammerhead cleavage.\textsuperscript{107, 108} An intriguing part of the experiment is the slow hammerhead activity seen even after addition of 10 mM [Co(NH$_3$)$_6$]$^{3+}$. At this stage only one Mn$^{2+}$ ion remains bound to the hammerhead (A9/G10.1) and this is a slow but active hammerhead ribozyme carrying out site-specific cleavage.

The metal ion binding site, the nature of the metal bound (inner sphere or outer sphere) and the number of metal ions involved in the cleavage reaction remain elusive in spite of decades of work in the field. Our experimental design and their outcome throw some light on the issues mentioned above. The hammerhead motif used in our study is the
same as the one crystallized by Pley$^{91}$ and coworkers and used by DeRose et al$^{84}$ for their experiments.

3.2 Experimental

3.2.1 Materials and Methods.
Hammerhead Ribozyme (34 nucleotides) and the substrate strand labeled at its 5’ end with fluorescein (13 nucleotides) were purchased from Dharmacon Research as deprotected, desalted and PAGE purified oligomers and used directly without further purification. The buffer 50 mM HEPES, 50 mM NaCl, pH 7.4 was prepared in autoclaved nano pure water, chelex treated to remove divalent metal ion contaminants and filter sterilized before use. No RNase contamination was detected. MgCl$_2$, MnCl$_2$, [Co(NH$_3$)$_6$]$^{3+}$ were purchased from Sigma and used without further purification. The fac- [Cr(NH$_3$)$_3$(H$_2$O)$_3$](CLO$_4$)$_3$ was synthesized from known literature procedures.$^{109,110}$

3.2.2 Kinetics with Mg$^{2+}$ or Mn$^{2+}$ ions:
The ribozyme and substrate were heated at 90$^\circ$C for 2 min and then slowly cooled to room temperature over a period of 20 min to ensure hybrid formation. The reaction was carried out in a volume of 25 µL, with 5 µM substrate, 20 µM ribozyme, and the appropriate concentration of metal ions (0-10 mM for Mg$^{2+}$, and 0-1 mM for Mn$^{2+}$).
Aliquots (4.5 µL) at specified time intervals were removed from the reaction stopped by addition of loading buffer (7 M urea, 0.5 M EDTA, 50% v/v glycerol) and placed on ice. Dyes were not added to prevent quenching of the flourescein. Prior to loading on a gel the samples were incubated in boiling water bath for 2 min to denature the substrate and product. The reaction was monitored by 15% PAGE, visualized with a Gel doc system under an uv-transilluminator and the substrate and product were quantified. The change in concentration of the substrate and product were plotted versus time and fit to a first order decay equation to obtain the k<br>obs for the reactions under consideration.

### 3.2.3 Kinetics in the presence of Mg^{2+} or Mn^{2+} and complex ions:

Reactions were carried out in the presence of 0.5 mM complex ions i.e. cobalt hexaammine [Co (NH$_3$)$_6$]$_3^+$ (1) and fac-chromiumtrisaquotriammine per chlorate fac-[Cr (NH$_3$)$_3$ (H$_2$O)$_3$] (CLO$_4$)$_3$ (2) with increasing concentrations of divalent ions (0-5 mM for Mg$^{2+}$ and 0 - 0.5 mM Mn$^{2+}$). The substrate and enzyme strand were heat annealed as mentioned earlier and incubated at 37$^{0}$C prior to addition of trivalent (complex) ions 1 or 2 (Figure 3.2) followed by divalent ions of appropriate concentration. The reactions were worked up as mentioned above and the kinetic parameters measured.
3.3 Results and Discussion

Mn\(^{2+}\) titration into hammerhead ribozyme and substrate (hybrid) shows an initial lag phase and the rate does not pickup unless all the high affinity sites are completely occupied (Figure 3.2). This happens at around 0.2 mM of Mn\(^{2+}\) concentration. The documented K\(_d\)'s for Mn\(^{2+}\) by EPR are ~ 4 \(\mu\)M and ~ 120 \(\mu\)M for the high and low affinity sites. This lag phase was observed in our system as well. The data was fit to a two metal ion-binding model, which yielded two sets of K\(_d\)'s for Mn\(^{2+}\) binding. The equations used to derive the fitting equation are
R + M₁ ⇌ RM₁ \hspace{2cm} K_a (1) = [RM₁] / ([R] [RM₁]) \hspace{1cm} (1)

RM₁ + M₂ ⇌ RM₁M₂ \hspace{2cm} K_a (2) = [RM₁M₂] / ([RM₁] [M₂])\hspace{1cm} (2)

Where R is the ribozyme and M₁ and M₂ are the same metal ions binding to site 1 and 2.

R₀ = R – RM₁ – RM₁M₂ \hspace{1cm} (3)

k_{obs} = k [RM₁M₂] \hspace{1cm} (4)

Rearranging equations 1, 2 and 3 substituting in 4 gives

k_{obs} = k K_a (1) K_a (2) [M₁] [M₂] [R] / (1 + [M₁] K_a (1) + [M₁][M₂] K_a (2))

This final equation was used to fit the data obtained from Mn^{2+} and Mg^{2+} catalyzed hammerhead reactions. The K_d’s obtained from this activity profile reflect those that are relevant to metal ion induced hammerhead catalysis. Binding of metal ions to the hammerhead that do not result in direct activity are not picked up by this method. The high affinity sites for Mn^{2+} observed by Horton et al are not seen in our experimental setup.
Figure 3.3: A plot of $k_{\text{obs}}$ versus divalent metal ion concentration for the hammerhead cleavage reaction. (A) Mn$^{2+}$ titrations of the hammerhead ribozyme. (B) Mg$^{2+}$ titration into the hammerhead. Fitting equations considers two interacting sites on the ribozyme.
Figure 3.4: A plot of $k_{obs}$ versus divalent metal ion concentration for the hammerhead cleavage reaction. Mg$^{2+}$ titration into the hammerhead, the fitting is carried out using a one binding site equation. Observed $K_d$ is 5.27 mM.

The model used here considers interacting sites and requires the binding of both metal ions for activity. The initial lag phase is more dominant in case of Mn$^{2+}$ than in Mg$^{2+}$. In case of Mg$^{2+}$, the fitting resulted in similar $K_d$’s whereas with Mn$^{2+}$, two different $K_d$ values were obtained 0.265 mM and 3.0 mM.

The difference in behavior of Mn$^{2+}$ and Mg$^{2+}$ leads us to believe that the mechanism of cleavage in the presence of Mn$^{2+}$ is different as compared to Mg$^{2+}$. Mn$^{2+}$ shows a more pronounced lag phase. The rates supported by the two are also remarkably different.
Manganese(II) with predominantly inner sphere interactions, shows higher affinities for the hammerhead. The rate profiles are also distinct. The two metal ion mechanism proposed for Mn$^{2+}$ supported catalysis need not hold for Mg$^{2+}$. The concentration of Mn$^{2+}$-OH species compensates for the difference in activity, but does not necessarily imply that Mg$^{2+}$ follows the two metal ion mechanism. Thus differences in the binding mode for the metal ions (inner sphere versus outer sphere), binding affinities and activity profiles all suggest that the two metals are distinct and that conclusions drawn for one need not, and will not always be applicable to the other.

DeRose and coworkers have reported two sets of Mn$^{2+}$ binding sites with 4 µM and 120 µM binding affinities. Approximately 8-9 Mn$^{2+}$ ions are estimated to be bound to the hammerhead. Addition of cobalt hexaammine (10 mM) to a hammerhead reaction with 0.5 mM Mn$^{2+}$ results in a drop in the rate of cleavage and the inhibition constant for cobalt hexaammine obtained is $\sim$ 22 µM. At these concentrations there is a residual rate for the hammerhead cleavage, which is intriguing and unexplained. DeRose and coworkers see a Mn$^{2+}$ bound to the hammerhead under these conditions.$^{105,106,111}$ We saw the same effect when 1 was added to a hammerhead reaction with 0.5 mM Mn$^{2+}$. This experiment when reversed with Mn$^{2+}$ or Mg$^{2+}$ added into 0.5 mM 1, gives the apparent K$_d$ of the divalent ion for the site it occupies by displacing 1 (Table 3.1).
Table 3.1: Apparent binding constants for the divalent ions in the presence of 0.5 mM complex ions 1 and 2.

<table>
<thead>
<tr>
<th>K_d (Mm) [Mn^{2+}]</th>
<th>K_d (mM) [Mg^{2+}]</th>
<th>K_d (mM) [Mn^{2+}]</th>
<th>K_d (mM) [Mg^{2+}]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.054 ± 0.009</td>
<td>2.73 ± 0.4</td>
<td>0.108 ± 0.009</td>
<td>2.90 ± 0.3</td>
</tr>
</tbody>
</table>

The observed rate constant increases on addition of divalents and a leveling of the k_{obs} is seen for limited addition of divalent ions (Figure 3.5). The data when fit to a one site-binding model gives apparent K_d’s for the divalent ions. The K_d’s suggest that the added divalent ion goes into the high affinity site and the complex ion occupies the low affinity site. The K_d’s obtained here are slightly higher than the ones obtained earlier, which is as expected since the K_d’s published earlier are direct binding constants whereas the ones obtained here are apparent binding constants.
Figure 3.5: (A) Titration of Mg$^{2+}$ into, 5 µM substrate + 20 µM ribozyme + 0.5 mM 1 or 2. (B) Titration of Mn$^{2+}$ into, 5 µM substrate + 20 µM ribozyme + 0.5 mM 1 or 2.

The maximal rate constant supported by this system is the same as when the reaction is reversed i.e. addition of 1 to reactions with 0.5 mM Mn$^{2+}$. The residual rate supported by 1 when it occupies the low affinity site implies that it is the catalytic site. The complex ion does not support catalysis to an extent supported by the divalent ion hence the drop in activity on its addition. A residual rate supports this conclusion. The rate constant
supported by the complex ions is different in both cases (1 and 2), which reinforces the above model. Complex 1 supports a lower rate, which is expected since it lacks the necessary ligands to support hydrogen-bonding interactions, which maybe required for the stabilization of the catalytic site or the transition state. Complex 2 on the other hand has three water ligands that can engage in hydrogen bonding interactions and its effect is seen in the rate constant that it supports. The apparent K_d for divalent ions in the presence of 2 is also higher since 2 competes more effectively with the divalent ion for binding in the catalytic site. The presence of water ligands increases the affinity of the complex ions for the hammerhead. This should result in more inhibition and the rate constant should drop further which is not the case. The complex ions 1 and 2, support hammerhead activity even in the absence of divalent ions (Table 3.2).
Table 3.2: Rate constants as measured for the complex ions 1 and 2 in the absence of divalents. N/A, Concentrations higher than 10 mM for 2 and 15 mM for 1 were found to substantially quenched the fluorescence of the substrate and could not be used for activity measurements.

<table>
<thead>
<tr>
<th>[Co(NH₃)₆]³⁺ (mM)</th>
<th>k₁obs (min⁻¹)</th>
<th>fac-[Cr(NH₃)₃(H₂O)₃]³⁺ (mM)</th>
<th>k₂obs (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.004</td>
<td>5</td>
<td>0.0345</td>
</tr>
<tr>
<td>10</td>
<td>0.0762</td>
<td>10</td>
<td>0.0563</td>
</tr>
<tr>
<td>15</td>
<td>0.0991</td>
<td>15</td>
<td>N/A</td>
</tr>
</tbody>
</table>

The concentrations required are higher and the rate constants are lower. This drop in activity with the trivalent ions, which have higher charge density may be due to the lack of water molecules in the coordination sphere. Also the fact that they support cleavage implies that the mechanism of action for the divalent ions is via an outer sphere mechanism.

The similar behavior of the two divalent ions in the presence of 1 and 2 suggests that the key differences in binding and activity come from the catalytic site. In our experimental
setup the catalytic site is already occupied and thus the binding of the divalent ions in the structural site is controlled exclusively.

Our experiments thus suggest that addition of 1 or 2 to hammerhead is not inhibitory and the added complex ion occupies the low affinity catalytic site. The divalent ion remains in the high affinity site and the two together support hammerhead activity. The fact that 2 supports a slightly higher rate as compared to 1 suggests slower catalysis and not inhibition. The presence of coordinated water molecules increases the rate of cleavage probably by stabilizing hydrogen-bonding interactions. These results also suggest that a metal hydroxide species may not be required for catalysis. The binding of the divalent ion in the high affinity site may lead to structural changes, which are required for catalysis.

3.4. Conclusions.

In conclusion we propose that the hammerhead has two sets of sites one, which serves a structural role and the other a catalytic role. As seen previously the structural site has to be occupied before catalysis can be initiated in case of Mn$^{2+}$. In the case of Mg$^{2+}$ there is no incubation period and the $K_d$’s of the structural and catalytic sites as evidenced from activity measurements are identical. The role of the structural site maybe less important in case of Mg$^{2+}$ as against Mn$^{2+}$. 
REFERENCES


(23) Young, E. T., II; Sinsheimer, R. L. **1964**, *10*, 562-564.


(41) Ohtsuki, K. I., N. *Journal of Antibiotics* 1975, 28, 229-236.


